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Age-related formaldehyde interferes with DNA methyltransferase function, causing memory loss in Alzheimer's disease

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ABSTRACT

Hippocampus-related topographic amnesia is the most common symptom of memory disorders in Alzheimer's disease (AD) patients. Recent studies have revealed that experience-mediated DNA methylation, which is regulated by enzymes with DNA methyltransferase (DNMT) activity, is required for the formation of recent memory as well as the maintenance of remote memory. Notably, overexpression of DNMT3a in the hippocampus can reverse spatial memory deficits in aged mice. However, a decline in global DNA methylation was found in the autopsied hippocampi of patients with AD. Exactly, what endogenous factors that affect DNA methylation still remain to be elucidated. Here, we report a marked increase in endogenous formaldehyde levels is associated with a decline in global DNA methylation in the autopsied hippocampus from AD patients. In vitro and in vivo results show that formaldehyde in excess of normal physiological levels reduced global DNA methylation by interfering DNMTs. Interestingly, intrahippocampal injection of excess formaldehyde before spatial learning in healthy adult rats can mimic the learning difficulty of early stage of AD. Moreover, injection of excess formaldehyde after spatial learning can mimic the loss of remote spatial memory observed in late stage of AD. These findings suggest that aging-associated formaldehyde contributes to topographic amnesia in AD patients.

1. Introduction

The hippocampal region of the human brain is widely considered to play a critical role in both the encoding and the retrieval of topographic memory (Maguire, 1997). Topographic amnesia is one of the most common symptoms of memory disorder in Alzheimer's disease (AD) patients and is characterized by the inability of either finding one's way around familiar environments, or in learning how to navigate in a new environment (McCarthy et al., 1996). Experimental evidence from animal studies supports this notion, namely that a healthy and intact hippocampus is essential for the formation of the so-called recent spatial memory, as well as for the maintenance of long-time memory (Clark et al., 2005; Eichenbaum et al., 1999). For example, hippocampal lesions can ultimately lead to a deficit in spatial memory in rats (Martin et al., 2005). Not surprisingly, hippocampus atrophy associated with topographic amnesia can be observed in AD patients (Li et al., 2007).

Recent studies have revealed that DNA methylation, which is regulated by enzymes with DNA methyltransferase (DNMT) activity, is a critical step required for memory formation in the brain (Day and sweatt, 2010; Levenson et al., 2006). It was shown that simultaneous knockout of DNMT1 and DNMT3a results in a reduction in global DNA methylation, as well as in deficits in both memory acquisition and retrieval in mice (Feng et al., 2010; Miller et al., 2010). Notably, overexpression of DNMT3a in the

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hippocampus can reverse spatial memory deficits in aged mice (Oliveira et al., 2012; Su and Tsai, 2012). In contrast, global DNA methylation, DNMT expression, and DNMT activity have been found to be decreased in brains of the aged mice (Oliveira et al., 2012), aged rats (Pogribny and Vanyushin, 2010), and in particular of autopsied brain samples taken from AD patients (Chouliaras et al., 2013; Mastroeni et al., 2010; Mastroeni et al., 2011). Thus, defects in global DNA methylation because of misregulated DNMT function may underlie hippocampal-related spatial memory deficits.

Previous evidence reveals that excess formaldehyde can induce amyloid aggregation (Chen et al., 2007; Kazachkov et al., 2007), tau aggregation, and hyperphosphoryation in vitro and in vivo (Lu et al., 2013; Nie et al., 2007). Formaldehyde scavenger, aminoguanidine can scavenge formaldehyde and block formaldehyde-induced amyloid aggregation (Kazachkov et al., 2007). These data indicate that endogenous formaldehyde participates in the pathologic process of AD. Our previous study showed that levels of brain formaldehyde in healthy mice or rats are within the range of 0.2–0.4 mM (physiological levels), similar to levels previously reported (Heck et al., 1982; Tong et al., 2011). Urine formaldehyde concentration in healthy human (0.02–0.04 mM) is about 10% of brain formaldehyde levels in autopsied samples (Takeuchi et al., 2007). Changes in urine formaldehyde concentration may reflect the fluctuation of endogenous formaldehyde in brains (Tong et al., 2013b). Interestingly, hippocampal formaldehyde increases to 0.5 mM in aged rats, and intrahippocampal injection of 0.5 mM formaldehyde impairs long-term potentiation and formation of spatial memory in healthy adult rats (Tong et al., 2013a). Moreover, formaldehyde in excess of normal physiological levels induces a reduction in global DNA methylation both in vitro and in vivo (Tong et al., 2013a). Here, we investigate how aging-associated accumulation of formaldehyde interferes with DNMT expression and activity. Further, we investigate the potential mechanisms of how this disturbance affects spatial memory.

2. Methods

2.1. Healthy volunteers (n = 139)

Healthy humans participated in the Mini Mental State Examination (MMSE) test (Ghanbari et al., 1998) and provided urine samples for collection. The participants were assigned into one of 3 categories: the adult age group (20–35 years old [28.3 ± 7.2], n = 48 (man/women = 23/25)); the old age group (65–78 years old [71.4 ± 7.1], n = 53 (man/women = 25/28)); and the advanced age (older) group (80–90 years old [84.2 ± 5.8], n = 38 (man/women = 21/17)). Morning urine samples were taken before breakfast. Participants were tested to have neither neurologic disorders nor known alcohol or drug abuse issues.

2.2. Clinical participants (n = 236)

The degree of dementia in AD patients without renal disease was scored as described previously (Fillenbaum et al., 1996) (Supplementary Table 1). Healthy humans with normal cognition (n = 55, clinical dementia rating [CDR] score = 0), patients with mild cognitive decline (n = 38, CDR score = 0.5), AD patients with varying degrees of dementia (n = 143, 1 ≤ CDR score ≤3) underwent a standardized clinical assessment including a medical history, a physical and neurologic examination together with CDR scale laboratory tests, a psychometric evaluation, an electroencephalograph, and a brain magnetic resonance imaging. All AD patients met the criteria set by the National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) for probable dementia on clinical grounds. Informed consent was obtained from all participants and/or relatives. This clinical investigation (SYXK2010-127) was approved by the Ethics Committee at the Beijing Hospital for the elderly individuals, China.

2.3. Autopsy sample of human

Autopsy hippocampus tissues from young, adult, healthy aged-matched controls with ApoE$^{\varepsilon 3/\varepsilon 3}$, and AD patients with ApoE$^{\varepsilon 4/\varepsilon 4}$ genotypes were provided by the Netherlands Brain Bank. Briefly, Alzheimer's disease patients (n = 9; age: 85.14 ± 5.62 years; ApoE genotypes: ε4/ε4; postmortem interval: 8.11 ± 5.62 hours; brain pH: 6.56 ± 0.06; amyloid: +++). Age-matched controls (n = 8; age: 83.60 ± 7.13 years; ApoE genotypes: ε3/ε3; postmortem interval: 8.25 ± 4.85 hours; brain pH: 6.52 ± 0.07; amyloid: −−). There were no significant differences between the groups with respect to demographic data.

2.4. Animal samples

Experiments involving Sprague-Dawley (SD) rats of different ages at 3, 16, and 30 months were in accordance with the Guide for the Care and Use of Laboratory Animals of National Institute of Health (China) and were approved by the Biological Research Ethics Committee of the Institute of Biophysics, Chinese Academy of Sciences (Beijing, China).

2.5. Reagents

All reagents were purchased from Sigma (USA) unless stated otherwise.

2.6. Human SH-SY5Y cells

Human SH-SY5Y cell lines were cultured as described previously (Tong et al., 2013a). The cultured cells were treated with different concentrations of formaldehyde (0, 0.15, 0.3, and 0.5 mM), 5-aza-2-deoxycytidine (5-AzA-CdR, 30 μM), or procainamide (PCA, 2 mM), before being scraped and processed for protein detection.

2.7. Rat training for formation of recent spatial memory

Three groups of the healthy male adult SD rats (200–250 g, n = 8 per group) were given bilateral intrahippocampal injections of excess formaldehyde (0.5 mM, 1 μL, over a period of 5 minutes) or 5-AzA-CdR (60 μM, 1 μL, over a period of 5 minutes) for consecutive 7 days, 30 minutes before the daily behavioral experiments, following a procedure as described previously (Tong et al., 2013a). Morris water maze trials to test for maintenance of spatial memory (90-second-probe test) were carried out on day 7. The rats that stayed in the target quadrant for longer than in all other quadrants were considered to have formed recent spatial memory. The treated hippocampi were then collected from the sacrificed rats for measuring formaldehyde and global DNA methylation levels. Three groups of rats with identical treatment were used for detecting expression of hippocampal DNMTs. A sham group was home caged without drug injection or spatial training, maintained in cages at room temperature (25 °C) under an alternating 12-hour light and/or dark cycle (lights on at 7 AM), with ad libitum access to food and water.

2.8. Rat training for formation of remote spatial memory

One group of normal adult SD rats (200–250 g, n = 8) received spatial training once a day and was given bilateral

intrahippocampal injections of normal saline (1 μL, over a period of 5 minutes) from day 31–37. Another 3 groups of normal adult SD rats (200–250 g, n = 8 per group) received training twice daily from day 1 to day 7 and then were given bilateral intrahippocampal injections of normal saline (1 μL, over a period of 5 minutes, group 1), excess formaldehyde (0.5 mM, 1 μL, over a period of 5 minutes, group 2), or PCA (5 mM, 1 μL, over a period of 5 minutes, group 3) from day 31–37. Probe trials were carried out on day 7 and day 37. The rats, which on day 37 stayed for a similar length of time in the target quadrant during the trials for maintenance of spatial memory as observed on day 7, were considered to have maintained successfully their remote spatial memory over the course of 30 days (the rats whose spatial memory abilities were below this standard were removed). The hippocampi were collected for measuring hippocampal formaldehyde and global DNA methylation levels. Three groups of rats with identical treatment were used for detecting hippocampal DNMT expression and activity. A sham group was home caged and neither drug injected nor spatially trained.

2.9. Detection of formaldehyde by Fluo-HPLC

All hippocampus samples were collected and immediately placed on ice before being stored at −70 °C until further processing. After centrifugation (3000 rpm, 4 °C, 10 minutes), supernatant fractions from brain homogenates (weight ratio of brain tissue: ultrapure water = 1:4) were used for analysis of formaldehyde by high-performance liquid chromatography with fluorescence detection (Fluo-HPLC) as described before (Luo et al., 2001; Tong et al., 2011).

2.10. Morris water maze behavioral test

Acquisition and maintenance of spatial memory by the trained rats was assessed by using a Morris water maze test as described previously (Morris, 1984; Tong et al., 2013a).

2.11. Quantification of global DNA methylation (5-mC)

A Methylamp Global DNA Methylation Quantification Ultra Kit (Epigentek, USA) was used to quantify global DNA methylation of genomic DNA in the hippocampus of SD rats according to the manufacturer's instructions.

2.12. DNMT activity assay

Nuclei were extracted from rat hippocampi using an EpiQuik nuclear extraction kit (Epigentek). DNMT activity was assayed using a DNMT assay kit (Epigentek) according to the manufacturer's instructions.

2.13. Western blotting of DNMTs

Electrophoretic analysis and Western blotting detection of target proteins in human SH-SY5Y cells and hippocampi (rats or human) were performed as previously described (Desplats et al., 2012; Tryndyak et al., 2006) using the following antibodies for Human SH-SY5Y cells: DNMT1 (1:1000; CST [5032], 200 kD), DNMT3a (1:1000; CST [3598], 130 kD), DNMT3b (1:250; Abcam [ab13604], 110 kD), β-actin (1:10,000; Sigma [A2228], 42kD); for hippocampi of SD rats: DNMT1 (1:500; Abcam [ab13537], 183 kD), DNMT3a (1:250; Abcam [ab13888], 102 kD), DNMT3b (1:1000; Abcam [ab13604], 110 kD), β-actin (1:10,000; Sigma [A2228], 42 kD); for autopsied hippocampi from AD patients and age-matched controls: DNMT1 (1:250; Abcam [ab13537], 183 kD), DNMT3a (1:250; Abcam [ab13888], 102 kD), DNMT3b (1:250; Abcam [ab13604], 110 kD), GAPDH (1:3000; Beyotime [AG019], China, 36 kD).

2.14. DNMT1 activity assay in vitro

DNMT1 activity was assayed using an EpiSeeker DNMT1 Inhibitor Screening Assay Kit (Abcam, USA) according to the manufacturer's instructions. The purified DNMT1 protein was purchased from Sigma (USA).

2.15. Data and statistical analyses

We determined the statistical significance of the in vivo data by analysis of variance followed by unpaired Student t tests. Morris water maze test was analyzed using 2-way analysis of variance. The statistical significance level was set at $p < 0.05$. Data are reported as means ± standard errors.

3. Results

3.1. Aging-associated formaldehyde accumulation induces cognitive decline via DNA methylation in human

3.1.1. Cognitive decline and formaldehyde accumulation in the normal aging human

To investigate the relationship between endogenous formaldehyde levels and cognitive decline during normal aging, we measured formaldehyde concentration in human urine using Fluo-HPLC, and assessed the cognitive abilities of healthy aged volunteers using the MMSE. The results showed that urine formaldehyde concentration in the older volunteers (n = 38, 80–90 years old, 0.045 ± 0.001 mM) was markedly higher than that in the adult volunteer group (n = 48, 20–35 years old, 0.031 ± 0.003 mM) (Fig. 1A). Furthermore, there was a significant cognitive decline in the older volunteer group (MMSE scores: 23.21 ± 0.61) compared with the adult volunteer group (MMSE scores: 28.33 ± 0.52) (Fig. 1B). Meanwhile, urine formaldehyde concentrations correlated negatively ($r = -0.998$) with the MMSE scores in the healthy aged volunteers (Fig. 1C). This result is consistent with emerging reported data (Yu et al., 2014).

3.1.2. Reduced global DNA methylation observed in the aging human hippocampus

To explore the relationship between hippocampal formaldehyde and global DNA methylation during human aging, we measured the average formaldehyde concentration and global DNA methylation levels from autopsied hippocampal samples of humans with different ages. The results showed that hippocampal formaldehyde concentration was markedly elevated in the older group (n = 9, 0.412 ± 0.031 mM) compared with the adult group (n = 6, 0.276 ± 0.011 mM) (Fig. 1D). We found that urine formaldehyde concentrations in the volunteers were about 10% of hippocampal formaldehyde levels detected in the autopsied samples. All these data suggest that accumulated hippocampal formaldehyde (from 0.20 to 0.45 mM) in the healthy aging human (from 70 to 80 years old) correlates with cognitive decline (MMSE scores: from 27 to 23). Moreover, global DNA methylation was significantly decreased in the older cohort (n = 9, 35.22% ± 0.71%) compared with the adult group (n = 6, 42.44% ± 1.02%) (Fig. 1E). Meanwhile, hippocampal formaldehyde concentration correlated negatively ($r = -0.949$) with global DNA methylation levels in these autopsied hippocampal samples (Fig. 1F).

3.1.3. Decline in DNMT expression levels in the aging human hippocampus

Because DNMT1 and DNMT3a play a key role in memory formation and maintenance (Feng et al., 2010), we measured DNMT1 and DNMT3a expression level in autopsied hippocampal samples from healthy humans of various ages. The results showed that both DNMT1 and DNMT3a (except DNMT3b) were markedly decreased in the older advanced age group (80–90 years old) compared with the adult human (20–35 years old) (Fig. 1G–I). This result supports the previous observation that DNMT expression levels decline during the aging process (Oliveira et al., 2012).

3.1.4. Downregulation of DNMTs in the presence of excess levels of formaldehyde

To address our hypothesis that the decline in DNMT expression during aging is because of age-related formaldehyde accumulation, we cultured human SH-SY5Y cell lines and treated the cells with different concentrations of formaldehyde and then measured DNMT protein expression levels. Previous studies have shown that 5-AzA-CdR inhibits expression and activity of DNMT1 and DNMT3 in vitro (Schneider-Stock et al., 2005). In this study, we found that 5-AzA-CdR (as a drug control) treatment for 24 hours clearly inhibited DNMT1 and DNMT3a expression but did not affect DNMT3b in cultured human SY5Y cells (n = 6, Fig. 1J–L). Similarly, excess levels of formaldehyde also induced a marked reduction in DNMT1 and DNMT3a expression levels in vitro (n = 6, Fig. 1J–L).

3.2. Age-related formaldehyde accumulation induces learning difficulty in spatial memory by reducing DNA methylation

3.2.1. Spatial memory decline and formaldehyde accumulation in aging rats

To verify our observation that formaldehyde-dependent inhibition of human DNMT expression also occurs in aging rat brains, we first explored whether age-related memory deficits are related to formaldehyde accumulation and global DNA methylation decline. The results showed that formaldehyde concentrations in the hippocampus, blood, and liver were all significantly increased in rats at 30 months of age compared with those in 3-month-old rats (Fig. 2A–C). Formaldehyde concentrations in the hippocampus and liver were also higher in 30-month-old rats compared with those of 16-month-old rats. Furthermore, there was a clear spatial memory deficit present in the 30-month-old rats compared with the 3-month-old ones (Fig. 2D–F). Together with Fig. 1, these results demonstrate that formaldehyde concentration correlates with cognitive capability in multiple species.

3.2.2. Decrease in global DNA methylation levels in the hippocampus of aging rats

To assess the relationship between aging and global DNA methylation, we measured global DNA methylation levels in rats at different ages. The results revealed that the global levels of DNA methylation in the hippocampus of (33.40% ± 1.14%) 30-month-old rats were significantly decreased relative to those in 3-month-old rats (38.73% ± 1.03%) (Fig. 2G). Furthermore, hippocampal formaldehyde concentrations correlated negatively ($r = -0.992$) with global DNA methylation levels in these rats at different ages ($p < 0.01$, Fig. 2H).

3.2.3. Decline in DNMT expression in the hippocampus of aging rats

To explore whether age-related formaldehyde induces a decline in global DNA methylation during the aging process in rats, we measured the protein expression levels of DNMT1, DNMT3a, and DNMT3b by Western blotting. The results showed that compared with 3-month-old rats, DNMT1 and DNMT3a levels were significantly reduced in 30-month-old rats (n = 8, Fig. 2I–L). In comparison, DNMT3b expression levels were not significantly changed between the 2 groups of rats (Fig. 2I–L).

3.3. Exogenous formaldehyde mimics learning difficulty in spatial memory in healthy adult rats

3.3.1. Deficits in retrieval of recent memory in rats injected with formaldehyde

To provide direct evidence that the observed deficits in spatial memory in aged rats as well as memory impairments in aged human is because of age-related formaldehyde accumulation, we injected formaldehyde at pathologic levels (0.5 mM) directly into the hippocampus of healthy adult rats before commencing spatial training sessions (Fig. 3A–E). Because inhibition of DNMT activity by 5-AzA-CdR blocks memory formation (Levenson et al., 2006; Miller and Sweatt, 2007), we injected 5-AzA-CdR (as a drug control) into the hippocampus of normal adult rats before spatial training. The results showed that the injection of the DNMT inhibitor also induced a deficit in spatial memory formation during the 7-day course of our behavioral test (n = 8, Fig. 3F and G). Similarly, application of exogenous formaldehyde not only impaired the ability for spatial learning from day 3 to day 6 (Fig. 3F) but also damaged the spatial memory retrieval ability of these rats (Fig. 3G).

3.3.2. Decline in DNMT expression in formaldehyde-injected rats

To test whether excess formaldehyde reduces global DNA methylation levels, we compared global DNA methylation and DNMT expression levels in rats. The results showed that formaldehyde or 5-AzA-CdR injection induced a marked decrease in global DNA methylation in these rats when compared with the control group (n = 8, Fig. 3H). Moreover, both DNMT1 and DNMT3a proteins exhibited a clear decrease in expression in the hippocampi of these 2 groups of drug-treated rats compared with the control group (Fig. 3I–K). However, DNMT3b expression displayed little change in the 30-month-old aged rats relatively to the 3-month-old young rats (Fig. 3I–L).

3.4. Excess levels of formaldehyde correlate with the degree of dementia in AD patients

3.4.1. Hippocampal formaldehyde accumulation in AD patients

To test our hypothesis that age-related formaldehyde accumulation induces amnesia observed in AD patients, we measured formaldehyde levels in autopsy samples of AD and age-matched controls. The results showed that concentrations of urine formaldehyde were higher in participants with abnormal cognition (n = 236, 0.042 ± 0.003 mM, CDR scores ≥0.5) compared with those of the control patients with normal cognition (n = 55, 0.029 ± 0.002 mM, CDR scores = 0) (Fig. 4A and Supplementary Table 1). Moreover, urine concentrations of formaldehyde from AD patients with dementia III (n = 47, 0.052 ± 0.005 mM) were also higher than those of the control subjects (n = 55, 0.029 ± 0.002 mM) (Fig. 4B). More importantly, urine formaldehyde levels were correlated positively ($r = 0.933$) with the severe degrees of dementia in these AD patients (Fig. 4C). Furthermore, hippocampal formaldehyde levels in the autopsied samples from AD patients (n = 8, 0.48 ± 0.02 mM) were higher than those of age-matched controls (n = 9, 0.41 ± 0.03 mM) (Fig. 4D). We found that urine formaldehyde concentrations in AD patients were about 10% of hippocampal formaldehyde levels in the autopsied samples from AD patients. All these results support the notion that the higher concentrations of hippocampal formaldehyde are associated with severe cognitive deficits.

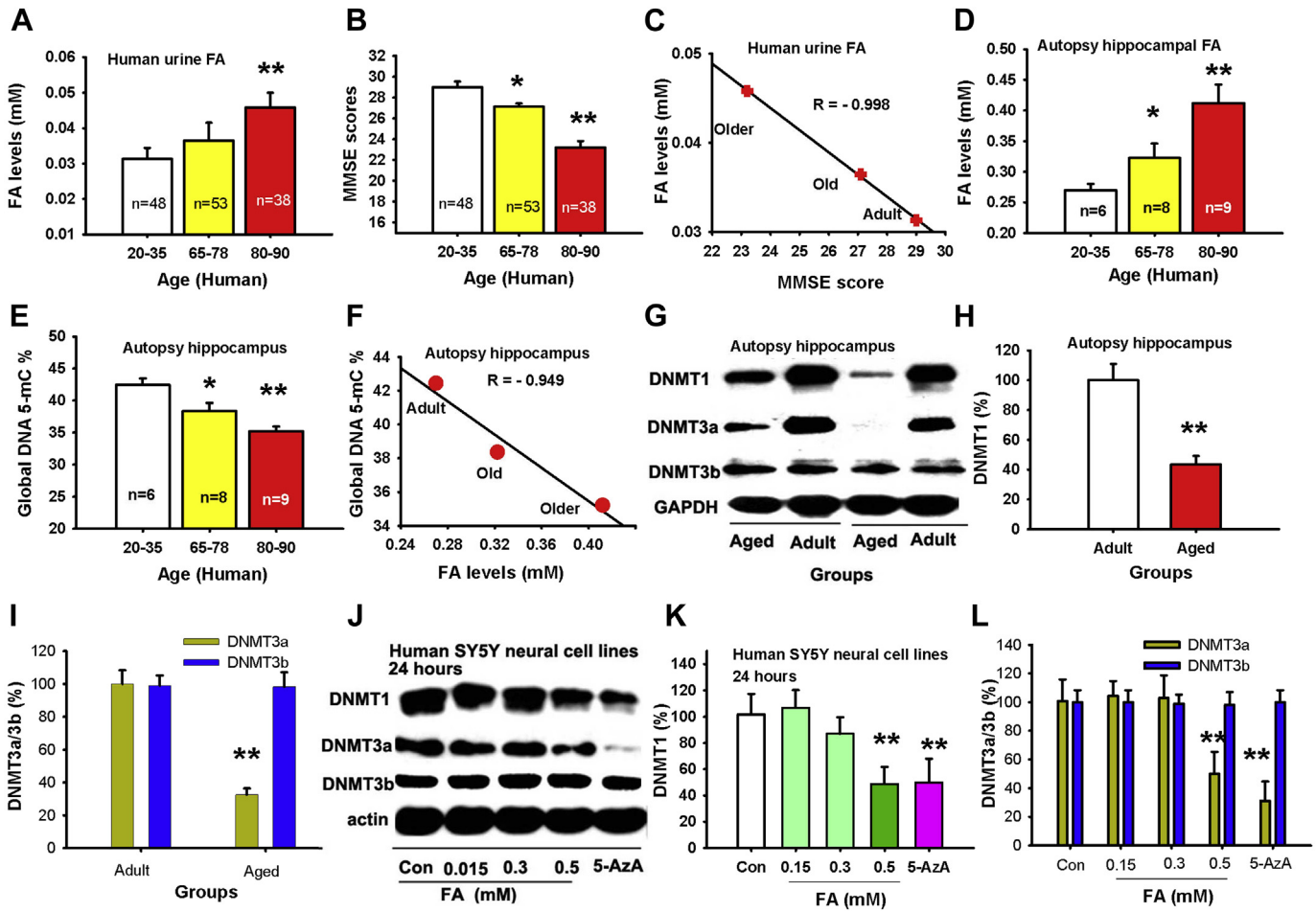

Fig. 1. Accumulation of endogenous formaldehyde and decline of global DNA methylation during human aging. (A) Urine formaldehyde accumulates with increasing age in humans. (B) Decline in human cognitive abilities evaluated by the mini mental state examination (MMSE). (C) Urine formaldehyde level is negatively correlated with the MMSE score. (D and E) Hippocampal formaldehyde accumulation and decline of global DNA methylation in autopsied hippocampal samples. (F) Hippocampal formaldehyde is negatively correlated with global DNA methylation levels. (G, H, and I) Aging affects DNMT1, DNMT3a, and DNMT3b expression. (J, K, and L) Formaldehyde and 5-aza-2-deoxycytidine (5-AzA-CdR, 30 μM) affects DNMT1, DNMT3a, and DNMT3b expression in human SY5Y cell lines within 24 hours. Abbreviation: DNMT, DNA methyltransferase (* $p < 0.05$; ** $p < 0.01$).

3.4.2. *Decline of DNMT expression levels in autopsied hippocampi of AD patients*

To investigate how formaldehyde reduces global DNA methylation level in AD patients, we measured global DNA methylation and DNMT protein expression levels. Our results revealed that there was a significant decrease in global DNA methylation levels in the AD patients (n = 8) compared with the control subjects (n = 9) (Fig. 4E). Moreover, there was a significant decrease in both DNMT1 and DNMT3a (except DNMT3b) proteins levels in the AD patient group (n = 8, 85.14 ± 5.62 years old) compared with those of the control group (n = 9, 83.60 ± 7.13 years old) (Fig. 4F–H). Notably, the level of reduction in DNMT1 expression (Δ = 66.65% ± 10.36%) was more severe than that for DNMT3a (Δ = 57.44% ± 4.34%) (Fig. 4F–H).

To provide direct evidence for our hypothesis that the decline in DNMT expression in AD patients is because of abnormally high concentrations of formaldehyde, we used cultured human SY5Y cells that had received various concentrations of formaldehyde. We then measured DNMT protein levels by Western blot. Given that PCA functions as a special inhibitor of DNMT1 expression (Lee, 2005), it was used as a control for DNMT1 function. The results showed that PCA markedly inhibited DNMT1 expression, but it did not affect DNMT3a or DNMT3b expression in vitro (n = 6, Fig. 4I–K). However, excess formaldehyde induced a significant decrease in DNMT1 and DNMT3a expression in vitro (n = 6, Fig. 4I–L).

3.5. *Excess levels of exogenous formaldehyde administered to healthy adult rats mimics topographic amnesia observed in AD patients*

3.5.1. *The loss of remote memory in rats with spatial training following injection of formaldehyde*

To mimic the abnormally high concentrations of hippocampal formaldehyde that is observed in AD patients with amnesia, we first trained adult SD rats for formation of 30-day stable spatial memory, and then examined whether injection of formaldehyde at the pathologic level (0.5 mM, based on our observation in autopsied hippocampal samples taken from AD (Fig. 4D), induces loss of stable spatial memory. Our results revealed that these rats injected with formaldehyde or PCA did not have a change in time of escape latency when compared with the control rats injected with saline (n = 8, Fig. 5A, B, E, and F). This indicates that there was no difference in the ability of spatial learning among these 3 groups. However, when probe trails in the Morris water maze were carried out on day 37, the rats that had been trained twice every day spent a longer time in the target quadrant than the rats trained only once daily (Fig. 5G). Markedly, the rats trained only once daily had less

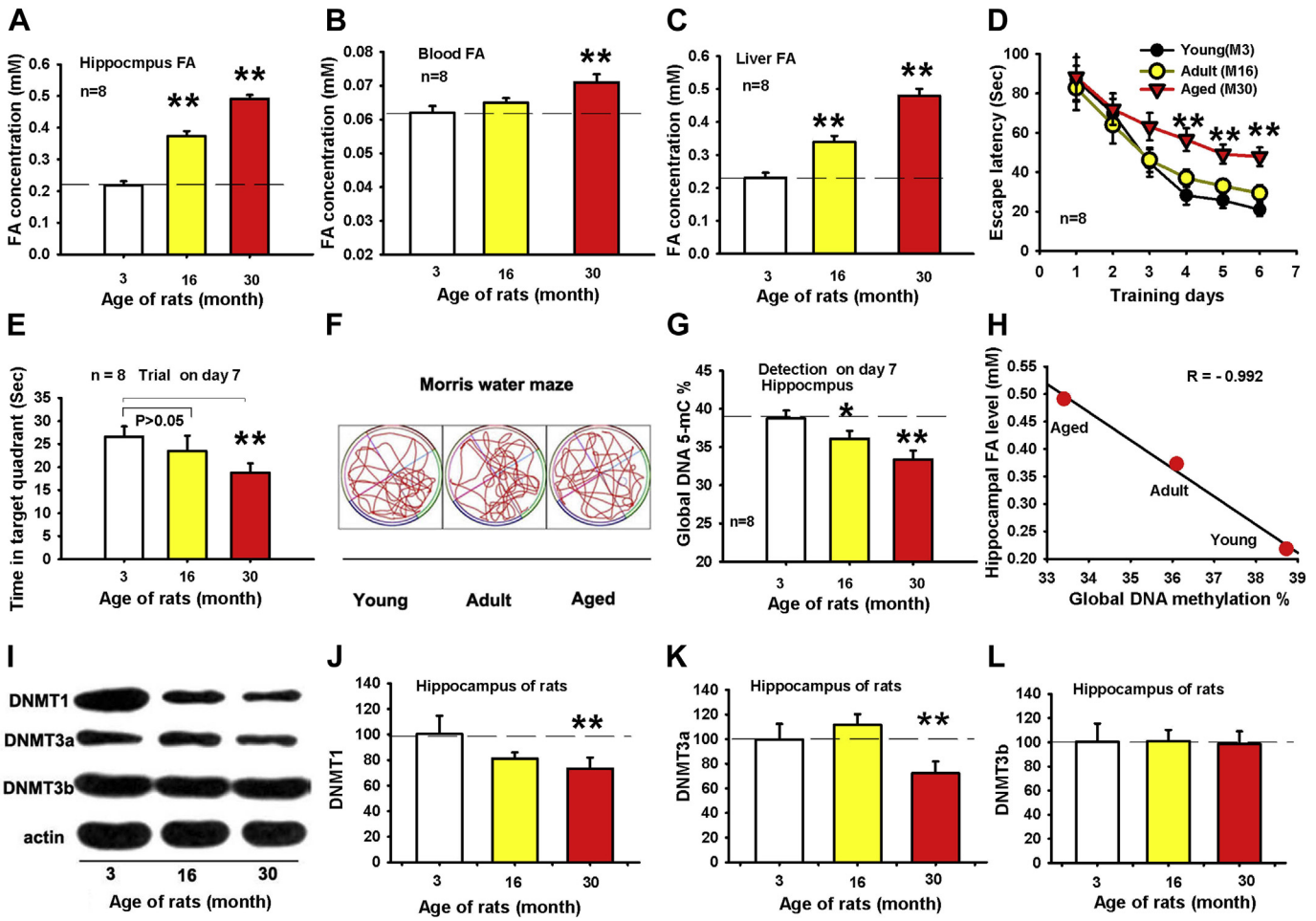

Fig. 2. Accumulation of endogenous formaldehyde and decline in global DNA methylation during aging in rats. (A, B, and C) Endogenous formaldehyde accumulates in the hippocampus, blood, and liver of aged rats. (D and E) Escape latency of spatial training and time in the target quadrant. (F) Swimming tracks in the Morris water maze. (G) Global DNA methylation in the hippocampus. (H) Hippocampal formaldehyde is negatively correlated with global DNA methylation level. (I, J, K, and L) Aging affects DNMT1 and DNMT3a but not DNMT3b expression in rats. Abbreviation: DNMT, DNA methyltransferase (* $p < 0.05$; ** $p < 0.01$).

time of staying in the target quadrant on day 37 compared with those rats who received training twice every day (Fig. 5G). These data indicate that rats trained twice every day have formed stable spatial memory on day 37.

In contrast, intrahippocampal injection of excess formaldehyde (0.5 mM) from day 30 to day 37 markedly reduced both swimming time and distance in the target quadrant (n = 8, Fig. 5C, G, and H). Swimming speed however was not affected as compared with the control rats (Fig. 5I). PCA, as a control drug, significantly reduced both the time and distance of swimming in the target quadrant of these rats on day 37 (n = 8, Fig. 5D, G, and H). Moreover, excess formaldehyde or PCA injection induced accumulation of hippocampal formaldehyde (n = 8, Fig. 5J).

3.5.2. Reduction in DNMT expression in rats with spatial training before injection of formaldehyde

To explore whether excess formaldehyde inhibits global DNA methylation in vivo, we measured the global DNA methylation and DNMT protein expression levels in PCA or formaldehyde treated rats. We found that excess formaldehyde or PCA injection markedly reduced global DNA methylation by day 37 and inhibited the activities of DNMTs in these rats when compared with healthy saline-treated rats (Fig. 5K and L). There was a significant increase in protein expression of DNMT1 and DNMT3a but not DNMT3b in rats that were trained twice daily compared with the control rats that were trained only once daily or with the shame rats, respectively (Fig. 5M–P). However, excess formaldehyde injection markedly inhibited DNMT1 and DNMT3a expression in vivo (Fig. 5M and O). PCA significantly repressed expression of DNMT1, but showed no effect on DNMT3a and DNMT3b expression (Fig. 5M and O). More importantly, the lower global DNA methylation, the more severe deficits of memory retrieval ability were observed in these rats with trained in Morris water maze (Fig. 5P). These results indicate that the inhibition of DNMT1 can induce loss of remote spatial memory.

4. Discussion

In this study, we found that accumulated endogenous formaldehyde is negatively correlated with cognitive abilities during human aging and positively correlated with severe degrees of dementia as observed in AD patients without renal disease. During aging, aging-associated accumulation of formaldehyde inhibits enzyme activity and protein expression of DNMT1 and DNMT3a, and thus blocks new formation of spatial memory (i.e., learning difficulty). In late stage of AD, chronic accumulation of hippocampal formaldehyde not only suppresses DNMT1 expression but also inhibits DNMT1 activity and induces loss of remote memory (Supplementary Fig. 1). These findings suggest that age-related

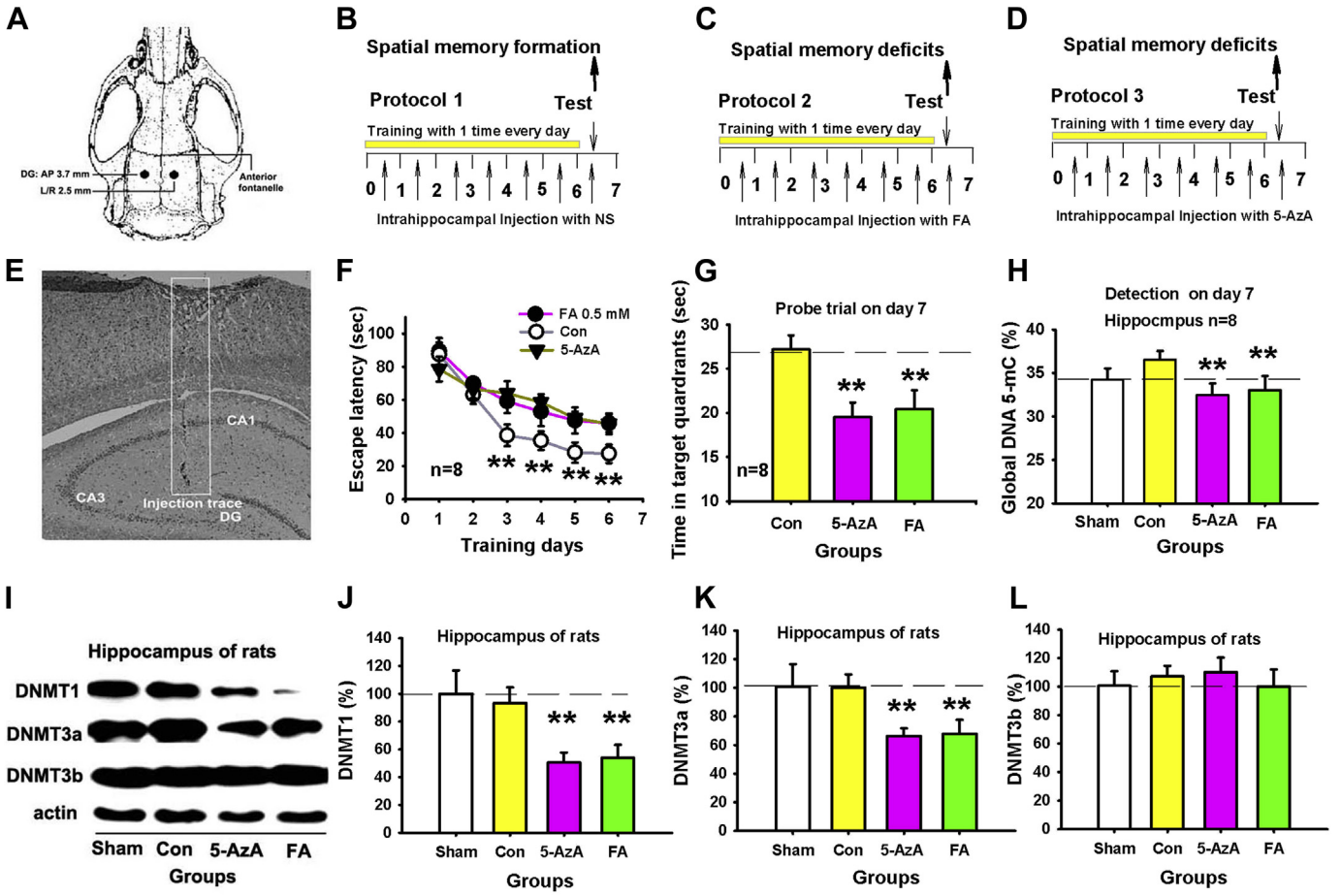

Fig. 3. Intrahippocampal injection of excess formaldehyde before spatial training in normal adult rats mimics learning difficulty in the early stage of AD. (A) Stereotaxic positioning in brains for bilateral intrahippocampal injection of drugs. (B–D) Protocols of rats injected with normal saline (NS), formaldehyde (FA, 0.5 mM) or 5-aza-2-deoxycytidine (5-AzA-CdR, 60 μM). (E) Injection tracks. (F and G) Escape latency of spatial training and time in the target quadrant. (H–L) Excess formaldehyde or 5-AzA-CdR affects DNMT1 and DNMT3a but not DNMT3b expression. Single yellow column in B–D represents single spatial training for 7 consecutive days. Abbreviations: AD, Alzheimer's disease; DNMT, DNA methyltransferase. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

formaldehyde is a critical factor during cognitive deterioration including topographic amnesia in AD patients (Budson and Price, 2005).

Decades of research have established that there are multiple factors that contribute to the endogenous accumulation of formaldehyde, which are (1) environmental pollution: mercury, paraquat, cycad toxins, and exogenous gaseous formaldehyde all lead to endogenous formaldehyde accumulation (Clejan and Cederbaum, 1993; Retfalvi et al., 1998; Spencer et al., 2012; Taranenko and Efimova, 2007); (2) disorders of formaldehyde-generating enzymes: activities of semicarbazide-sensitive amine oxidase (a blood formaldehyde-generating enzyme) are elevated in aged rats, as well as in patients suffering from diabetes and AD (del Mar Hernandez et al., 2005; Ferrer et al., 2002; Mercier et al., 2007); (3) deficiently of formaldehyde-degrading enzymes: deficiency of aldehyde dehydrogenase 2 (a nonspecific formaldehyde-degrading enzyme) induces memory loss and neurodegenerative disease (Ohta and Ohsawa, 2006). Aldehyde dehydrogenase 2 polymorphism was shown previously to correlate with the diagnosis of diabetes and AD (Suzuki et al., 2004; Wang et al., 2008). Alcohol dehydrogenase 3 (a specific formaldehyde-degrading enzyme, Km = 0.38 mM [Cinti et al., 1976]) can defend against neurodegenerative processes (Galter et al., 2003; Jelski et al., 2006; Mori et al., 2000). Also, it was shown that deficiency of alcohol dehydrogenase 3 in *Drosophila* results in loss of visual memory (Hou et al., 2011). Unsurprisingly, a pathologic level of formaldehyde (well above the concentration of physiological levels) was found in the hippocampi of different AD animal models, for example, in senescence-accelerated prone mice 8, APP-transgenetic mice, and APP/PS1-transgenetic mice (Tong et al., 2011). However, the precise mechanisms that allow pathologic levels of hippocampal formaldehyde to impair spatial memory remain to be elucidated.

Our novel findings described here suggest that specific DNMTs are one of the molecular targets during age-associated formaldehyde induced memory decline. Global DNA methylation is controlled by a highly regulated network of DNMTs (Jin et al., 2011). Thus, changes in expression of DNMTs have a direct impact on the dynamics of global gene expression patterns of a cell, usually resulting in reexpression of formerly silenced genes. Two of the DNMTs, namely DNMT1 and DNMT3a, are considered maintenance methyltransferases (Fatemi et al., 2002), and knockout of DNMT1 in mice was shown to have various physiological effects, including deficits in acquisition of spatial memory. In contrast, DNMT3a and DNMT3b are responsible for the establishment of new DNA methylation marks (Li et al., 2007). It was shown previously that mice which had formed fear memory also exhibited an increase in DNMT3a expression (Miller and Sweatt, 2007); however, there was a marked decrease in DNMT3a expression in AD model mice (Sierksma et al., 2013). In agreement

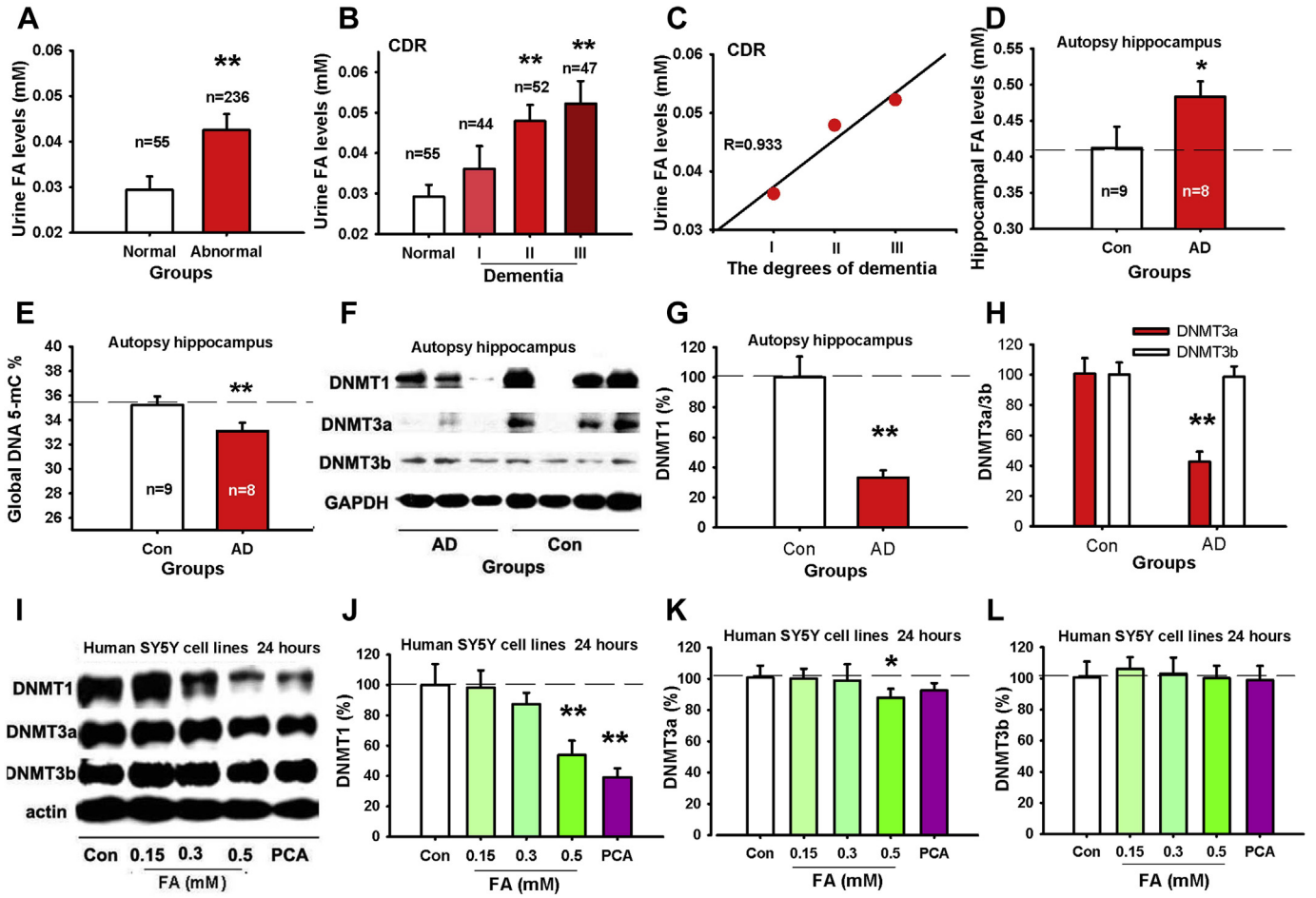

Fig. 4. Abnormal accumulation of endogenous formaldehyde and decline in global DNA methylation in Alzheimer's disease patients. (A) Urine formaldehyde accumulates in AD patients. (B) Degrees of dementia are evaluated by the clinical dementia ratio (CDR). (C) Urine formaldehyde levels are positively correlated with the CDR score. (D and E) Formaldehyde accumulation in the hippocampus and decline of global DNA methylation in autopsied hippocampal samples. (E) Hippocampal formaldehyde is negatively correlated with global DNA methylation levels. (F–H) DNMT1, DNMT3a, and DNMT3b expression are decreased in autopsy samples from AD patients. (I–L) Both formaldehyde and procainamide (PCA, 2 mM) affect DNMT1, DNMT3a, but not DNMT3b expression in human SY5Y cell lines within 24 hours. Abbreviations: AD, Alzheimer's disease; DNMT, DNA methyltransferase.

with this observation, virally induced overexpression of DNMT3a in the hippocampus of mice was reported to reverse age-related decline in memory (Oliveira et al., 2012; Su and Tsai, 2012). Together, these data along with our results support the finding that both DNMT1 and DNMT3a are required for the formation of new memory, through their overlapping roles in maintaining DNA methylation (Feng et al., 2010; Miller et al., 2010).

The finding that injection of formaldehyde at pathologic levels in rats with a stable spatial memory erases remote memory via inhibition of DNMT activity supports the notion that activity of DNMTs is essential for the maintenance of remote spatial memory. Activity of DNMTs has also been shown to regulate global DNA methylation levels (Jin et al., 2011). Another previous study revealed that blocking of DNMT activity by injection of DNA demethylating reagents (5-AzA-CdR, zebularine [zeb], and RG108), induces loss of remote memory in mice (Miller et al., 2010). The incorporation of 5-AzA-CdR into DNA leads to the irreversible binding of DNMT1 to incorporated 5-AzA-CdR residues and the rapid loss of DNMT1 activity (Christman, 2002). In this study, 5-AzA-CdR, a total DNMTs inhibitor, induced a dose-dependent inhibition of DNMT1 activity in vitro. As well as a specific inhibitor of DNMT1 enzyme-PCA (Lee, 2005), formaldehyde also elicited a dose-dependent decrease in the purified DNMT1 activity in vitro (Supplementary Fig. 2). These data further support that formaldehyde can reduce global DNA methylation levels by inhibiting DNMT1 activity. Formaldehyde can modify cystine residues of proteins (Metz et al., 2006). Both DNMT1 and DNMT3a contain a cystine-rich zinc-binding region (Bachman et al., 2001). Therefore, we speculate that formaldehyde inhibits activities of DNMTs by incorporating into cystine residues of DNMTs. To date, no studies have been conducted to investigate 5-AzA-CdR effects on the activity of DNMT3a and DNMT3b. Although 5-AzA-CdR has been found to downregulate DNMT1 and DNMT3a expression, not modulate DNMT3b expression, the exact mechanism of function is unclear (Schneider-Stock et al., 2005). Substantial evidence demonstrates that global DNA methylation and DNMT activity decrease in aged cells (Casillas et al., 2003; Lopatina et al., 2002), aged brains (Bollati et al., 2009; Liu et al., 2009, 2011), and especially in AD patients (Mastroeni et al., 2010; Mastroeni et al., 2011). In addition, DNMT1 is responsible for maintaining correct DNA methylation patterns (Milutinovic et al., 2004). We show that DNMT1 expression in autopsied hippocampal samples from AD patients was lower than that from the age-matched controls. This result is consistent with previous reports (Chouliaras et al., 2013; Desplats et al., 2011; Mastroeni et al., 2010). Moreover, the rats trained twice daily exhibited a marked upregulation of DNMT1 in

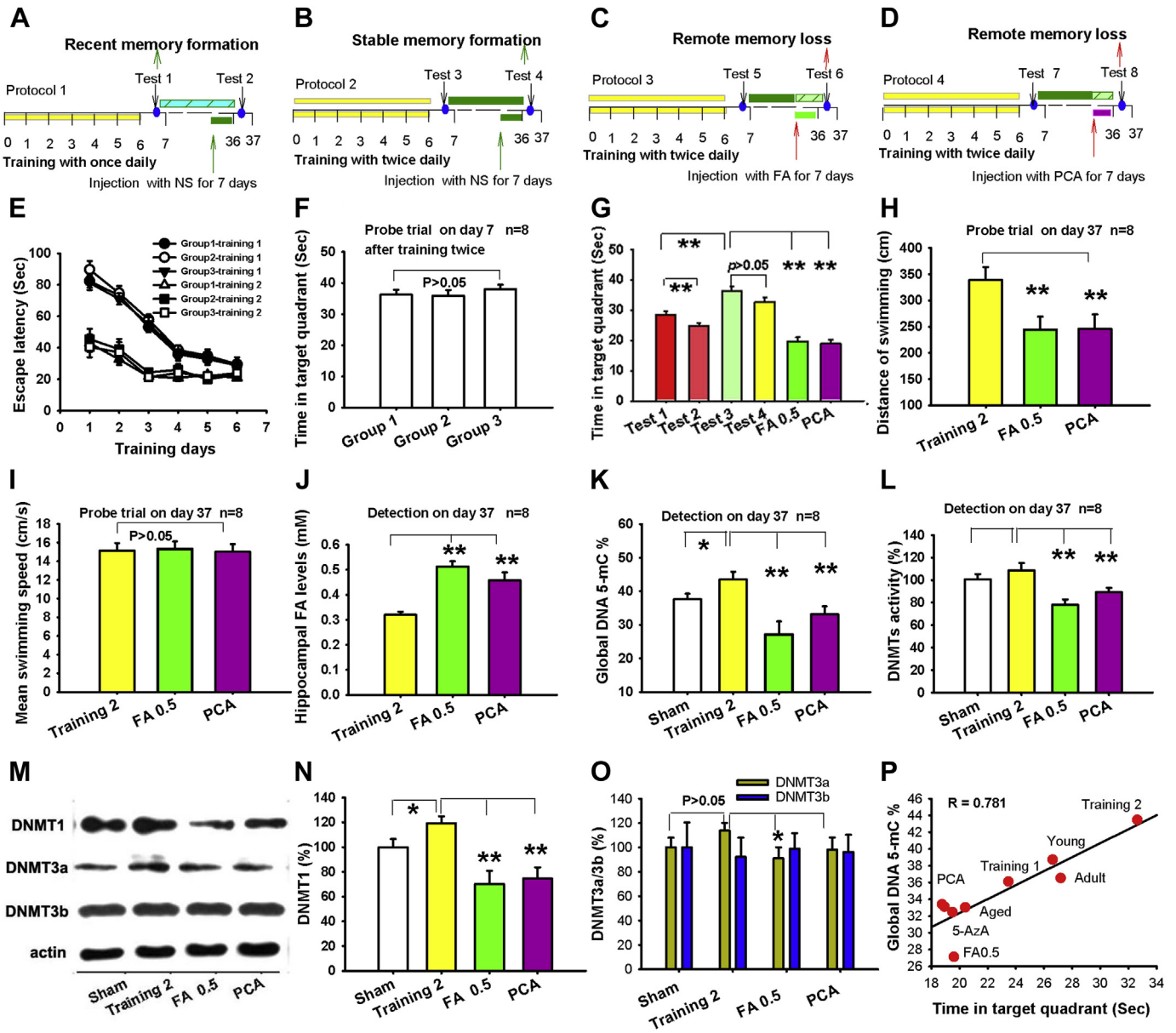

Fig. 5. Intrahippocampal injection of excess formaldehyde after spatial training in healthy adult rats mimics AD-related topographic amnesia (loss of remote spatial memory). (A–D) Protocols for injection with (1) normal saline (NS, training once daily); (2) normal saline (NS, training twice daily); (3) formaldehyde (FA, 0.5 mM, training twice daily); or (4) procainamide (PCA, 5 mM, training twice daily). (E–I) Escape latency, time in the target quadrant, swimming distances in the target quadrant, and the swimming speed in these rats. (J–L) Excess formaldehyde or procainamide (PCA) affects hippocampal formaldehyde, global DNA methylation, and DNMTs activity in the hippocampus. (M–O) Excess formaldehyde and PCA affects DNMT1 and DNMT3a but not DNMT3b expression. (P) Global DNA methylation levels were positively correlated with memory retrieval ability of rats. Single yellow column in A: rats with spatial training once daily for 7 consecutive days. Two yellow columns in B–D: rats with spatial training twice daily for consecutive 7 days. Training 2: the rats with spatial training twice daily without FA or PCA injection. Group training 1: rats with the first training for consecutive 7 days. Group training 2: rats with the second training for consecutive 7 days. Test 1, 2, 3, 4, 5, 6, 7, and 8: probe trail on different days as indicated. Abbreviations: AD, Alzheimer's disease; DNMT, DNA methyltransferase. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

the hippocampus. However, injection of PCA or excess formaldehyde inhibited DNMT1 expression and activity in rats, and thus induced loss of remote spatial memory. These data suggest that DNMT1 may play a more important role in maintaining remote spatial memory compared with DNMT3a.

A recent study shows that Tet1, a member of the ten-eleven translocation (Tet) family of methylcytosine dioxygenases which catalyze the oxidation of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) and promotes DNA demethylation is critical for memory extinction (Rudenko et al., 2013). During aging, a decrease in global hippocampal 5-mC level (Pogribny and Vanyushin, 2010) and an increase in 5-hmC content have been observed in the hippocampus (Chen et al., 2012). Compared with age-matched controls, a more severe decrease in global hippocampal 5-mC content and a greater increase in 5-hmC levels have been found in postmortem cortical tissue from AD patients detected by using immunohistochemistry (Mastroeni et al., 2010; Mendioroz Iriarte et al., 2014; van den Hove et al., 2012). These data suggest that aging enhances memory extinction and more severe memory deterioration occurs in late-stage AD patients. Thus, it is probable that formaldehyde-mediated global DNA methylation decline accelerates cognition deficits in AD patients. Although other studies

have observed an increase in 5-mC and 5-hmC detected by using immunofluorescence or immunohistochemistry (Condliffe et al., 2014; Coppieters et al., 2014), the detecting methods, ApoE genotypes, the sampling time, and the sampling regions from AD patients should be considered. Because postmortem intervals do indeed affect gene expression from human brain (Birdsill et al., 2011). In this study, the autopsied hippocampal samples from control and AD were strictly designed to carry with ApoE genotypes: ε3/ε3 and ε4/ε4. An emerging evidence shows that formaldehyde can induce ApoE4 aggregation (Rizak et al., 2014). In AD transgenic model mice, there was a marked different expression in DNMT3a, 5-mC, and 5-hmC in the DG, CA3, and CA1 subregion of the hippocampus (Sierksma et al., 2013). This result suggests that the accuracy and precision of the sampling regions affect the observed results in autopsied samples from AD patients. In conclusion, it is highly possible that inhibition of DNMT activity and protein expression by aging-associated formaldehyde underlie the pathophysiology of AD.

Disclosure statement

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2014.07.018.

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